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STRUCTURE AND SPECIFICITY OF T CELL RECEPTOR
 γ/δ ON MAJOR HISTOCOMPATIBILITY COMPLEX
ANTIGEN-SPECIFIC CD3⁺, CD4⁻, CD8⁻ T LYMPHOCYTES

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Most antigen-specific peripheral murine T lymphocytes express the T cell differentiation antigens, CD4 or CD8, and a TCR composed of a CD3-associated disulfide-linked heterodimer, TCR- α/β (1, 2). However, recent studies have identified a distinct population of CD3⁺, CD4⁻, CD8⁻ cells that appear early in thymic ontogeny and are maintained in the mature thymus and the peripheral lymphoid organs throughout adult life (3–5). Immunoprecipitation studies of murine CD3⁺, CD4⁻, CD8⁻ T cells have shown that the majority of these cells do not express cell surface TCR- α/β ; rather their TCR complex includes a 45-kD δ glycoprotein disulfide linked to either the TCR-V γ /C γ 1¹ gene product or the TCR-V γ /C γ 4 gene product (demonstrated by immunoprecipitation with anti-TCR- γ antisera) (5–7). These results suggested that the TCR- γ/δ ⁺ cells may compose a separate lineage that develops in the thymus before the TCR- α/β -expressing T cells (7).

If the TCR- γ/δ ⁺ T cells are a distinct lineage, then what is their function and specificity? One characteristic of TCR- α/β -bearing cells that distinguishes them from B lymphocytes is the skewing of their repertoire toward the recognition of MHC molecules (8, 9). MHC-restricted antigen recognition has been definitively attributed to the TCR- α/β heterodimer by experiments in which transfection and expression of TCR α and β chains of a given T cell clone confer both the clone's antigen specificity and MHC restriction specificity on the transfected cell (11, 12). Although the TCR- α/β heterodimers are distinct from the TCR- γ/δ heterodimers, these two types of TCR have structural characteristics in common. Like the TCR- α/β receptor (1, 2), the TCR- γ/δ receptors are heterodimers noncovalently associated with CD3 (3, 7, 13–16). In addition, both the TCR- γ and δ gene products are formed by in-frame rearrangements of distinct V, (D), and J gene segments during T cell development (17, 18). Interestingly, the gene elements encoding the TCR- δ gene are localized within the TCR- α gene complex, and rearrangements of V α segments to D δ

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¹ The TCR- γ nomenclature used in this manuscript was derived from Garman et al. (10).

and J δ have been described (19, 20). These structural similarities, as well as the presence of TCR- γ/δ -bearing T cells in the thymus, raise the possibility that TCR- γ/δ cells may also recognize MHC antigens. To address this question, we have generated alloreactive CD3⁺, CD4⁻, CD8⁻ TCR- γ/δ cell lines and clones. One such TCR- γ/δ cytolytic T cell (CTL) line was shown to recognize an MHC-linked antigen (21). The present studies were undertaken to examine the nature of the MHC-linked ligand of the TCR- γ/δ CTL line and clones and to analyze the biochemical and molecular nature of the TCR proteins expressed on the cell surface. We have also developed additional MHC-specific TCR- γ/δ CTL in order to begin to explore the diversity of receptor expression and the MHC molecule recognition by this T cell subset.

Materials and Methods

Animals. BALB/c and C57BL/10 (B10) *nu/nu* mice, 2–10 mo old, were purchased from the Frederick Animal Research Facility (Frederick, MD). All other inbred and congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or were bred in our own colony (National Institutes of Health, Bethesda, MD).

Monoclonal Antibodies and Antisera. The mAb 145-2C11, specific for the CD3- ϵ chain of the TCR complex, was derived in our laboratory (22). The anti-C γ 1/C γ 2 and anti-C γ 4 antisera were generated by injecting rabbits with synthetic peptides derived from published gene sequences as previously described (3,5).

T Cell Lines and Clones. The derivation and propagation of the MHC-specific CD3⁺, CD4⁻, CD8⁻ T cell lines has been described (21). Clones were derived from the lines by limiting dilution. Briefly, 2 d after stimulation, activated T cells were added to individual wells of 96-well flat-bottomed microtiter plates at 0.5 cells/well in the presence of 100 U/ml human rIL-2 (Lot LP315; Cetus Corp., Emeryville, CA; reference 23) and 5×10^5 irradiated (3,000 rad) allogeneic spleen cells. After 1–2 wk, cells from positive wells were transferred to 24-well plates with additional IL-2 and irradiated allogeneic stimulator cells. Thereafter the cloned cells were propagated in the same fashion as the T cell lines. CD3⁺, CD4⁻, CD8⁻ bulk TCR- γ/δ T cells were derived as described elsewhere (5). Erythrocyte-depleted, freshly isolated BALB/c splenocytes were suspended in complete medium (RPMI 1640 supplemented with glutamine, 10% FCS, 2-ME (5×10^{-5} M), Hepes buffer, and nutrient mix [sodium pyruvate, nonessential amino acids, glutamine, penicillin, streptomycin; NIH media unit]) at 10^7 cells/ml. Cells were incubated with anti-Lyt-2.2 (83-12-5, reference 24) and anti-L3T4 (RL 172.4, reference 25) mAb culture supernatants, each at a final concentration of 25%, for 30 min at room temperature. Cells were washed in complete medium and resuspended at 10^7 cells/ml in complete medium containing a preselected concentration of rabbit sera (as a source of complement). The cells were incubated for 30 min at 37°C and were washed again. Dead cells were removed by centrifugation on a Ficoll-Hypaque gradient (Lymphocyte M; Cedarlane Laboratories Ltd., Ontario, Canada). Viable cells were cultured for 2 d in the presence of anti-CD3 mAb followed by 3 d in IL-2-containing Con A-induced culture supernatants.

Lymphokine Assays. The secretion of soluble lymphokines was monitored by the ability of culture supernatants of activated T cells (collected after 24 h of incubation) to support the growth of the lymphokine-dependent HT-2 cell line (kindly provided by Dr. Richard Hodes, NIH). 5×10^3 HT-2 cells were cultured for 24–36 h with the supernatants to be assayed, then pulsed for 16 h with 1 μ Ci of [³H]thymidine. IFN- γ was assayed as previously described using an ELISA method (26).

Biochemical Analysis. Cultured alloreactive T cells were ¹²⁵I surface labeled by the lactoperoxidase method (3) and lysed in 1% digitonin buffer for anti-CD3 and anti-C γ 4 immunoprecipitations, or in 0.5% NP-40 lysis buffer for anti-C γ 1/C γ 2 precipitations as described (5). Lysates were precleared overnight at 4°C with protein A agarose beads and rabbit anti-eosin (coupled to KLH) antiserum (a kind gift from Dr. Pierre Henkart, NIH). Precleared lysates were immunoprecipitated with 50 μ l of anti-CD3 culture supernatant or 20 μ l of rabbit anti-TCR antisera and were analyzed by SDS-PAGE as described (3). Proteins were visual-

ized by drying the gels and developing with Kodak XAR-5 film in cassettes containing Lightning Plus developing screens (DuPont Co., Wilmington, DE), stored at -70°C . Approximate molecular weights of proteins were determined by comparison to Bethesda Research Laboratories (Gaithersburg, MD) or Amersham Corp. (Arlington Heights, IL) prestained high molecular weight standard markers that were electrophoresed in the same gels. *N*-glycosidase treatment and reduction/alkylation procedures were performed as previously described (5).

Northern and Southern Blot Analyses. Cellular RNA was prepared after extraction with guanidinium isothiocyanate (27). 10 μg /lane of RNA was subjected to electrophoresis on 1% agarose/0.7% formaldehyde gels at 100 V for 4 h before transfer to nitrocellulose filters. 10 μg of high molecular weight DNA was digested to completion with the indicated restriction enzymes according to the manufacturer's specifications and was subjected to electrophoresis on 0.8% agarose gels at 25 V for 36 h. Southern transfers, hybridization, and washes were performed as previously described (28, 29). Sizes of labeled DNA fragments were calculated from a Hind III digest of phage λ DNA.

cDNA Cloning and Sequencing. Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose (type 7; Pharmacia Fine Chemicals, Piscataway, NJ) chromatography (30). First and second strand cDNA synthesis, and cloning into phage $\lambda\text{gt}10$ were performed using kits supplied by Amersham Corp. according to the manufacturer's specifications. cDNA was size selected by chromatography over Sepharyl S-500, superfine (Pharmacia Fine Chemicals). The cDNA library was screened by phage filter hybridization using the *C δ* probe. DNA purified from individual positive recombinant plaques were digested with Eco RI and Klenow labeled with $\gamma\text{-}^{32}\text{P}$ -dATP (New England Nuclear, Boston, MA). DNA fragments were subcloned into M13Mp9 (31) and sequenced by the dideoxynucleotide chain termination method (32).

DNA Probes. The *V γ 1*, *V γ 2*, *V γ 3*, and *V γ 4* probes, all cloned into the pGEM II vector, were the generous gift of Dr. David Raulet (MIT, Boston, MA) (10). *V γ 1* is a 500-bp Eco RI-Hind III fragment; *V γ 2* is an 800-bp Eco RI-Pst I fragment; *V γ 3* is a 300-bp Eco RI fragment; and *V γ 4* is a 1.2-kb Hind III-Sph I fragment. The *C δ* probe, generously provided by Drs. Tom McMillen and John Coligan (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) is a 500-bp Eco RI fragment derived from a full-length cDNA clone. The *J δ 1* probe is a 3' genomic Eco RI-Hind III fragment cloned into pGEM3. The *J δ 2* probe is a 2.5-kb Eco RI-Xba I fragment cloned into the same vector. Both probes were kindly provided by Dr. Yueh-hsiu Chien, Stanford University, Stanford, CA (19).

Cell-mediated Cytolysis Assay. $1\text{--}5 \times 10^6$ target cells (Con A-induced splenic blasts) were radiolabeled with $^{51}\text{NaCrO}_3$ (300 μCi , Amersham Corp.) for 1 h at 37°C . Effector cells were harvested 1–2 d after removal of exogenous rIL-2 after stimulation, washed, resuspended in culture medium, and plated in 96-well U-bottomed microculture plates (Costar, Cambridge, MA) containing $1\text{--}2 \times 10^3$ radiolabeled target cells at selected E/T ratios. After a 4-h incubation at 37°C , the culture supernatants were harvested using the Titertek supernatant collection system (Skatron Inc., Sterling, VA). The percentage of specific lysis was calculated using the formula: Percent specific lysis = $100 \times [(\text{Experimental cpm} - \text{spontaneous cpm})/(\text{maximum cpm} - \text{spontaneous cpm})]$. Experimental cpm represents the ^{51}Cr released from target cells incubated with effector cells. Maximum cpm represents the release of radiolabel from target cells in a 0.05 N solution of HCL. Spontaneous cpm represents the background release of target cells cultured in media in the absence of effector cells.

Results

TCR- γ/δ CTL Can Recognize a Relatively Nonpolymorphic MHC-linked Class I Molecule. To identify potential ligands for T cells expressing TCR- γ/δ receptors, we derived an alloreactive T cell line from the peripheral lymph nodes of BALB/c (H-2^d) *nu/nu* mice stimulated with low density B10.BR (H-2^k) spleen cells (21). Phenotypic and biochemical analyses demonstrated that this cell line was CD3⁺, CD4⁺, CD8⁺ and expressed the product of a rearranged TCR-C γ 1 gene on its surface (21). Func-

tional analysis of the BALB/c *nu/nu*-derived T cell line showed that it recognized H-2^k-expressing but not H-2^d-expressing target cells, as judged by proliferation and cytotoxicity (21). When assayed for lymphokine production (Fig. 1), both H-2-congenic B10.BR and BALB.K stimulator spleen cells (APC) induced the production of IFN- γ and a lymphokine capable of supporting the proliferation of HT-2 cells. Interestingly, further analysis has shown that these γ/δ cells produce granulocyte/macrophage colony-stimulating factor but no detectable IL-2 or IL-4 (Cron, R. Q., T. F. Gajewski, S. O. Sharrow, F. W. Fitch, L. Matis, J. A. Bluestone, submitted for publication). In contrast, H-2-identical BALB/c and B10.D2 APC did not induce lymphokine production (Fig. 1). These results were consistent with the conclusion that these TCR- γ/δ CTL recognized an MHC-linked antigen. However, the BALB/c *nu/nu* anti-B10.BR TCR- γ/δ CTL line was broadly crossreactive, recognizing a variety of allogeneic MHC target cells including H-2^{k,b,f,q,s} (Fig. 2A). Of the panel of targets tested, the only allogeneic target cells not lysed expressed the H-2^p haplotype.

Two possibilities could explain the high degree of MHC crossreactivity. First, the CTL line might have been heterogeneous, comprising numerous distinct CTL clones each crossreactive for a limited subset of allogeneic targets. Alternatively, the individual TCR- γ/δ cells might recognize a relatively nonpolymorphic antigen expressed on either polymorphic or nonpolymorphic MHC gene products common to many MHC-disparate strains. To distinguish between these two possibilities, CTL clones were isolated from the CTL line by limiting dilution and were examined for TCR expression and specificity. Two representative clones, F4 and G8, were isolated and shown to be CD3⁺, CD4⁻, CD8⁻ based on flow cytometry (FCM) analysis (data not shown) and TCR- γ/δ expressing as shown by immunoprecipitation (Fig. 3). The G8 (Fig. 2B) and F4 (data not shown) clones were examined for cytolytic activity on various allogeneic targets and were found to have the same reactivity pattern as the parental line. Thus, the high degree of crossreactivity of the CTL clone indicated that these TCR- γ/δ cells recognized a relatively nonpolymorphic antigenic determinant.

To evaluate more precisely the nature of the MHC-linked molecule recognized by the TCR- γ/δ CTL line and clones, CTL specificity was further analyzed by sev-

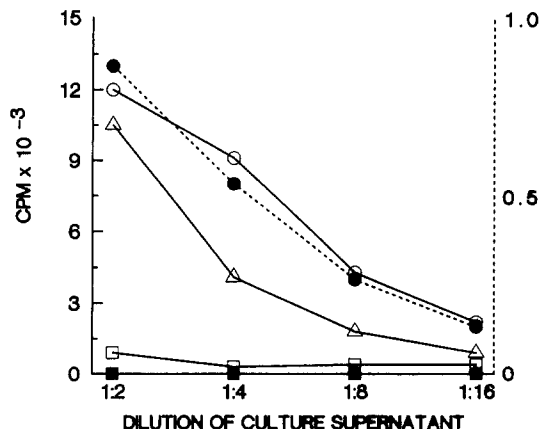


FIGURE 1. TCR- γ/δ CTL produce lymphokines in response to allogeneic antigen. Supernatants harvested 24 h after stimulation were incubated with lymphokine-dependent HT-2 cells or analyzed for IFN- γ production by ELISA. Solid lines represent induction of proliferation of HT-2 cells by supernatants from CTL stimulated with BALB/c (solid square), BALB.K (open circle), B10.D2 (open square), and B10.BR splenocytes (open triangle). Dotted lines represent IFN- γ in supernatants from cells stimulated with BALB/c (solid square) or B10.BR (closed circle) cells.

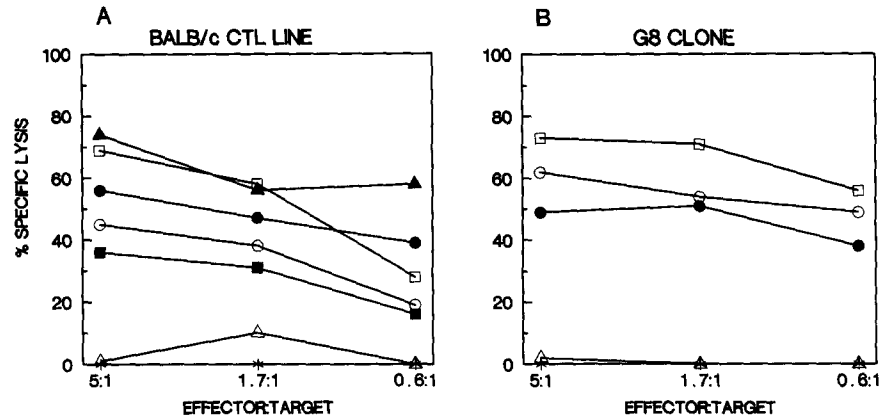


FIGURE 2. Specificity of BALB/c CTL line and G8 clone on allogeneic Con A blast target cells. Distinct target cells are denoted by symbols: *open squares*, C57BL/10 (bbbbbb); *closed triangles*, B10.M (fffff); *closed circles*, B10.S (sssss); *open circles*, B10.BR (kkkkk); *closed squares*, B10.Q (qqqqq); *open triangles*, B10.P (ppppp); and *asterisk*, B10.D2 (ddddd). The MHC haplotypes are designated in parentheses (K-I-D-Q-TL).

eral approaches. First, lysis of an H-2^k-expressing tumor cell line (R1.1) and its β_2 -microglobulin-negative (β_2 M⁻) variant (R1.E) was examined (Fig. 4 A). Although the CTL efficiently lysed the parental class I-expressing cell line, no lysis of the β_2 M⁻ mutant was observed. These results suggested that the TCR- γ/δ CTL recognize a β_2 M-associated antigen, most likely a class I MHC molecule.

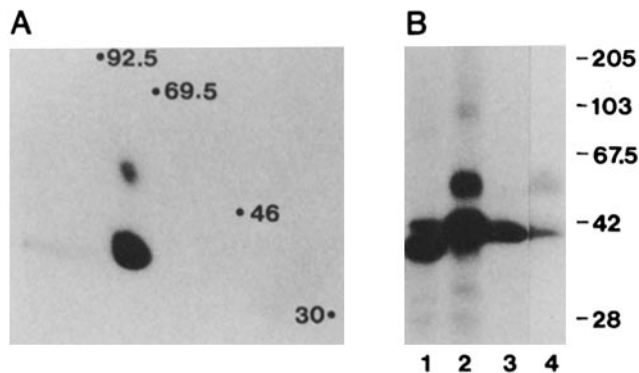


FIGURE 3. (A) Radiolabeled G8 cells were solubilized and immunoprecipitated with the anti-CD3 mAb and analyzed by two-dimensional (nonreducing conditions along the horizontal axis and reducing conditions along the vertical axis) off-diagonal SDS-PAGE gels. Molecular weights are noted as dots on the figure. (B) Anti-CD3 and anti-TCR- γ and anti-TCR- δ immunoprecipitates were *N*-glycosidase-treated or reduced and alkylated as described previously (5) and run on a one-dimensional gel. Lane 2 represents an anti-CD3 immunoprecipitated sample of G8, while lanes 3 and 4 represent samples reprecipitated with the anti-TCR- γ 1/C γ 2 antiserum (lane 3) or the anti-TCR- δ antiserum (lane 4) after reduction/alkylation. Lane 1 represents an anti-CD3 immunoprecipitate followed by *N*-glycosidase treatment.

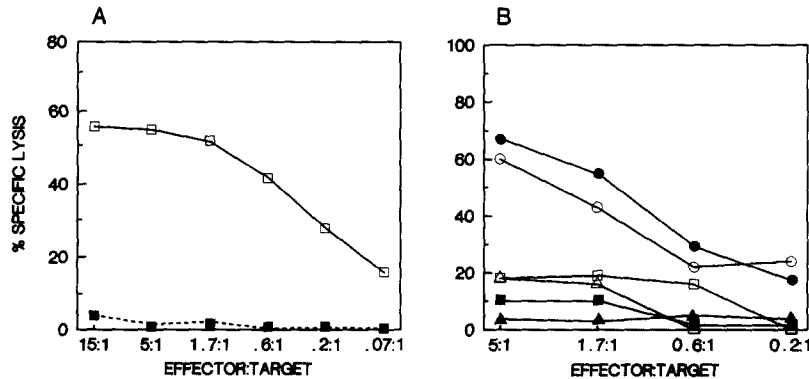


FIGURE 4. Analysis of TCR- γ/δ cell specificity on H-2⁻ and H-2-congenic target cells. (A) The lysis of the TCR- γ/δ CTL on R1.1 (open squares) and R1.E (closed squares) cells. (B) The lysis of targets from H-2^a recombinant mice; B10.Q (open circles), B10.AKM (open squares), B10.MBR (closed triangles), B10.SQR (closed squares), B10.QBR (closed circles), and B10.T(6R) (open triangles).

Next, a more detailed analysis of the MHC specificity of the CTL line was performed using target cells derived from recombinant, MHC-congenic inbred mice. Our previous studies localized the target antigen recognized by the TCR- γ/δ CTL to the D, L, Q, or TL regions of the MHC (21). For example, B10.OL (H-2K^d, D^k), but not B10.A (H-2K^k, D^d), were lysed by the BALB/c *nu/nu* anti-B10.BR TCR- γ/δ CTL line. Similar H-2D-end reactivity of the CTL was observed on the crossreactive H-2^b haplotype (21). Further analyses, performed in the present studies, demonstrated that the CTL were broadly crossreactive on target cells from recombinant H-2^a strains (Fig. 4 B). Although the CTL line lysed target cells from the B10.Q strain (H-2K^q, D^q), it did not lyse either B10.AKM (H-2K^k, D^q) or B10.T(6R) (H-2K^q, D^d) targets (Fig. 4 B). These results implied that either the CTL recognition required the expression of H-2^a genes encoded in both the K and D end of the MHC, or that the CTL recognized an MHC-linked molecule other than H-2K^q or H-2D^q. This latter possibility was supported by further analysis using other H-2^a recombinant strains, namely B10.MBR (H-2K^b, D^q), B10.QBR (H-2K^b, D^q), and B10.SQR (H-2K^s, D^q) (Fig. 4 B; reference 33). The CTL lysed the B10.QBR but not B10.SQR or B10.MBR target cells. Although all of the strains are H-2D^q, the TL genes of these mice are of different origins (33). Specifically, whereas the B10.Q and B10.QBR haplotypes express TLa^a (derived from the DBA/1 strain), the B10.AKM, B10.SQR, and B10.MBR express TLa^c (derived from an outbred mouse). Thus the data indicate that these CTL may recognize an MHC class I molecule encoded in the TL region of the TLa^a phenotype. This conclusion was supported by the finding that several anti-H-2D and Q region-specific mAbs did not inhibit CTL activity (data not shown). In addition, B10.M (H-2^f) cells are lysed by the CTL. This result was of interest in view of recent studies by Flaherty et al. (33a), who have shown that in the H-2^f haplotype (B10.M strain) the Q1 through Q9 genes have been deleted, including Q7, which is thought to encode the Qa-2 gene product. Thus, it would appear that the BALB/c *nu/nu* anti-B10.BR CTL line does not recognize a Q region-encoded gene product. Finally, an H-2^d haplotype pre-B cell line

(R8.15) transfected with the *H-2D^b* gene was not lysed by the H-2^k-specific BALB/c CTL (data not shown).

Biochemical Analysis of the TCR Expression on the MHC Antigen-specific TCR- γ/δ CTL. Detailed biochemical and molecular analyses of the TCR proteins expressed by these class I MHC antigen-specific TCR- γ/δ cells were performed in order to determine similarities or differences between these TCR- γ/δ cells and the predominant TCR- γ/δ populations described in the thymus and peripheral lymphoid organs. Initial biochemical analysis of the G8 cells suggested that the same predominant disulfide-linked TCR proteins were expressed on the clones and parental CTL line as on bulk TCR- γ/δ thymocytes (3, 7) (Fig. 3 A). To determine which molecular weight species represented the individual TCR- γ and TCR- δ proteins expressed on the G8 clone, the TCR proteins were immunoprecipitated with specific anti-TCR antisera after dissociating the chains by reduction and alkylation. As shown in Fig. 3 B, the anti-TCR-C γ 1/C γ 2 antiserum specifically reacted with the low M_r protein (35×10^3 ; lane 2 vs. 3), while the anti-TCR- δ antiserum preferentially reacted with the high M_r protein (45×10^3) (lane 2 vs. 4) of the 80×10^3 M_r heterodimer. The degree of *N*-linked glycosylation of the 35×10^3 M_r TCR γ protein was next examined by treating immunoprecipitated samples with *N*-glycosidase. Analogous to previous findings in the BALB/c and B6 strains of mice (3, 7), the TCR- γ protein immunoprecipitated by the anti-CD3 antibody was *N*-glycosylated (lane 1 vs. 2). *N*-glycosidase treatment reduced the M_r of the TCR- δ chain from 45 to 37×10^3 and the TCR- γ chain from 35 to 32×10^3 M_r . Therefore, this heterodimer apparently represents the previously described TCR-V γ 2C γ 1/ δ /CD3 complex that is expressed on the majority of CD3⁺,CD4⁻,CD8⁻ BALB/c fetal and adult thymocytes (3, 7).

Molecular Analysis of the TCR Proteins Expressed on the MHC Antigen-specific TCR- γ/δ CTL. Although initial *N*-linked glycosylation analysis suggested that the TCR- γ protein expressed on the BALB/c alloreactive TCR- γ/δ cells was derived from a rearranged *V γ -C γ 1*, the precise *V γ* gene expressed could not be assigned because *V γ 2*, *V γ 3*, and *V γ 4* have all been shown to rearrange to the *C γ 1* gene (10). To address this question, molecular analyses of TCR- γ genes expressed in the alloreactive TCR- γ/δ CTL were performed. Previously published Southern blot analysis of Eco RI-digested genomic DNA (10) have shown two predominant TCR- γ rearrangements using a γ constant region probe specific for C γ 1/C γ 2/C γ 3. A 16-kb rearranged fragment results from joining of the *V10.8A* (*V γ 1.2*) segment to the *J γ 2* segment, encoding a *V γ 1.2-C10.5* (*C γ 2*) protein, while a 17kb band represents a *V γ 2* gene rearrangement (*V γ 2-C13.4* (*C γ 1*)) (10). Fig. 5 illustrates a Southern blot analysis of Eco RI-digested genomic DNA from BALB/c liver and the alloreactive TCR- γ/δ CTL using *V γ* DNA probes. Upon Eco RI digestion of germline BALB/c liver DNA, two restriction fragments of 5.7 kb and 10.8 kb hybridize to the *V γ 1* probe, a 5.2-kb fragment hybridizes to the *V γ 2* probe, and 7-kb bands hybridize to the *V γ 3* and *V γ 4* probes (Fig. 5). Both the *V γ 3* and *V γ 4* genes remain in the germline configuration in the BALB/c TCR- γ/δ line and the F4 clone. In contrast, both the *V γ 1* and *V γ 2* genes have rearranged in the CTL line and clones.

In addition, both *V γ 1* and *V γ 2* were expressed as full-length TCR- γ mRNAs in the BALB/c *nu/nu* line and the G8 line (Fig. 6). However, *V γ 2-C γ 1* but not *V γ 1.2-C γ 2* encodes a glycosylated protein (34). Thus, from the combined biochemical and mo-

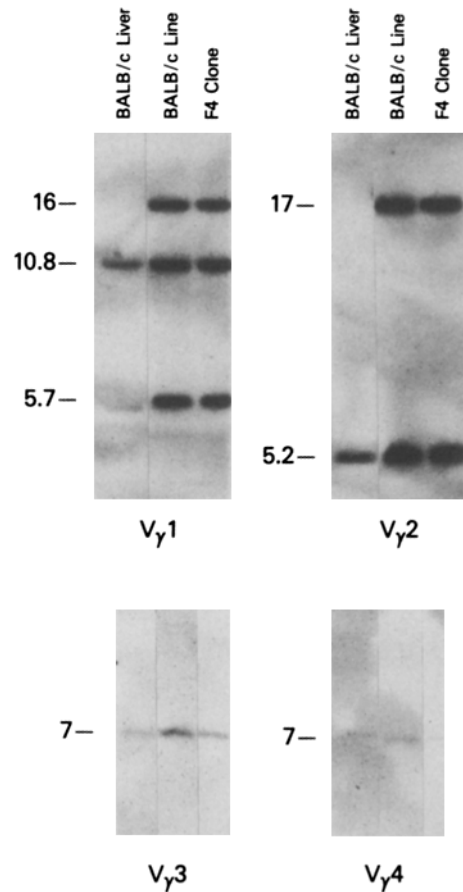


FIGURE 5. Southern Blot analysis of TCR- γ rearrangements in the TCR- γ/δ BALB/c *nu/nu* anti-B10.BR alloreactive T cell line and the F4 clone. Eco RI-digested DNA from liver (germline) and the T cell line and clone were hybridized to each of the four probes shown. Performance of the Southern analysis and the origin of the probes are described in Materials and Methods. The sizes of the hybridizing bands are shown (kb).

lecular biologic analyses, it can be concluded that the alloreactive BALB/c *nu/nu* line and clones express the TCR- γ protein product of a *V γ 2-C γ 1* gene.

Analysis of TCR- δ Gene Expression. TCR- δ mRNA expression in the MHC-specific line was confirmed using a C δ probe (Fig. 6). Messenger RNA species of 2.0 and 1.6 kb were observed in the BALB/c *nu/nu* anti-B10.BR TCR- γ/δ cells, consistent with previous reports (17). Moreover, truncated but not full-length TCR- α and TCR- β mRNAs were expressed (Fig. 6), which confirmed the exclusive expression of TCR- γ/δ in these cells. In contrast, no TCR- δ mRNA could be detected in a TCR- α/β expressing spleen cell population.

TCR- δ rearrangements in the alloreactive line and the G8 clone were next examined with two *J δ* probes (19) capable of detecting rearrangements to either of the two *J δ* gene segments, *J δ 1* or *J δ 2*, that have been identified (Fig. 7). Two predominant rearranged bands of 6.2 and 4.8 kb were observed after hybridization of Eco RI-digested DNA from the BALB/c line and G8 clone to the 3' *J δ 1* probe. For comparison, TCR- δ rearrangements were also examined in bulk TCR- γ/δ spleen cells, a BALB/c spleen-derived CD3⁺, CD4⁺, CD8⁻ T cell population propagated in vitro

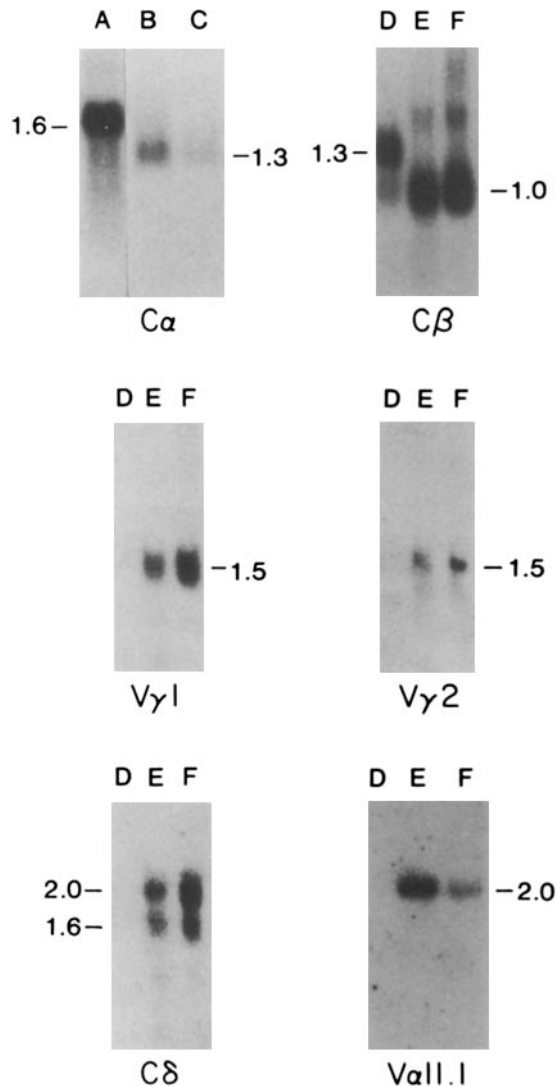


FIGURE 6. Analysis of TCR gene expression in TCR- γ/δ alloreactive T cells. RNA was prepared from the T cell population shown and was examined for the expression of the indicated TCR genes. The origin of the probes and the performance of the Northern analysis is described in Materials and Methods. The sizes of the RNA species were determined by using 5.1 kb and 2.0 kb for ribosomal 28S and 18S as markers. Equal loading of the RNA per lane was determined by ethidium staining of the gels. Lane *A* represents RNA from a cytochrome *c*-specific TCR- α/β Th cell, F1.A2. Lane *D* represents RNA from whole B10 spleen cells cultured with anti-CD3 as described in Materials and Methods. Lanes *B* and *E* represent RNA from the BALB/c *nu/nu* anti-B10.BR TCR- γ/δ CTL line, while lanes *C* and *F* represent RNA from the TCR- γ/δ clones F4 and G8, respectively.

for 1 wk by nonspecific stimulation with the anti-CD3 mAb 145-2C11, as described in the experimental procedures section (5). At least four *J δ 1* rearranged bands, including a predominant one of 7.4 kb, were observed in the bulk TCR- γ/δ cells. The 6.2-kb rearrangement of the alloreactive CTL but not the 4.8-kb rearrangement was also detected in the unselected TCR- γ/δ T cells. Analysis of the *J δ 2* locus failed to detect any rearrangements in either the alloreactive CTL or the bulk TCR- γ/δ T cells, consistent with the prior observation (19) that rearrangements to the *J δ 2* segment are far less frequent than to *J δ 1*.

To characterize the TCR- δ gene, a cDNA library was constructed in phage λ gt10 from the BALB/c *nu/nu* anti-B10.BR T cell line, and TCR- δ cDNA clones were iso-

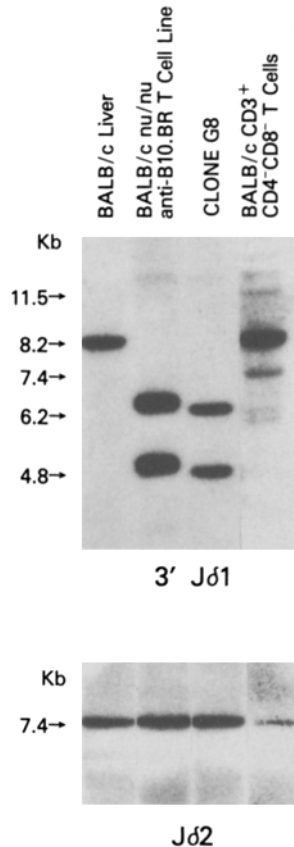
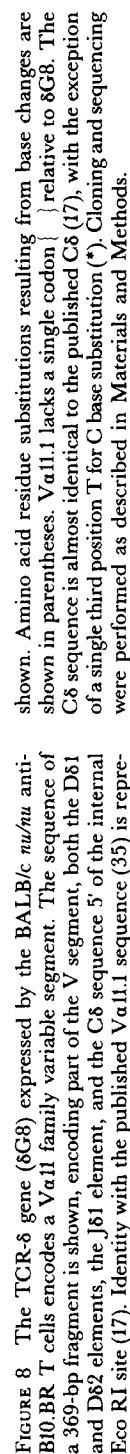


FIGURE 7. Southern Blot analysis of TCR- δ rearrangements in the BALB/c anti-B10.BR alloreactive TCR- γ/δ line. DNA from BALB/c liver, the BALB/c *nu/nu* anti-B10.BR TCR- γ/δ -expressing T cell line, the G8 clone, and a CD3⁺, CD4⁻, CD8⁻ BALB/c splenic T cell population propagated in vitro for 1 wk by stimulation with the anti-CD3 mAb was digested with Eco RI (*top*) or HINDIII (*bottom*) and hybridized to the 3'J δ 1 and J δ 2 probes, respectively. Southern analysis was performed as described in Materials and Methods. The sizes of the hybridizing bands are shown (kb).

lated using the *C δ* probe. Eco RI digestion of purified phage DNA from colonies containing putative TCR- δ inserts produced several recombinants consisting of a 0.9-kb fragment (the size of the cDNA fragment encoded by the *C δ* gene downstream of its internal Eco RI site and 3' untranslated sequence) (17) as well as one or more additional smaller fragments. Several of the fragments, most likely to contain both *C δ* and variable gene elements, were subcloned and sequenced. The sequence of a representative 369-bp fragment, δ G8, is shown in Fig. 8. The clone encodes 5' *C δ* sequence virtually identical to that previously published (17), followed upstream by J δ 1, D δ 2, and D δ 1. Assignments of J δ 1, D δ 1, and D δ 2 are based upon the published germline sequences (19, 20). N region nucleotides are present between D δ 2 and J δ 1, and between the V segment and D δ 1. The inclusion of both D δ 1 and D δ 2 gene segments is analogous to that observed in previously described TCR- δ cDNA clones derived from adult but not fetal thymocyte populations (19, 20). The putative V δ gene segment was distinct from any previously published unique V δ sequences derived from fetal or adult thymocytes cDNA clones (17, 19, 20), but was found to be >95% homologous at the DNA level to a previously reported V α gene segment designated V α 11.1 (35). The identity of V δ G8 with the V α gene family was also supported by the fact that the 5' end of the subcloned δ fragment represented the exact



site of an internal Eco RI site within the published *Va11.1* sequence (35). Moreover, a *Va11.1* V segment probe derived from an antigen-specific TCR- α/β -expressing T cell clone (28) hybridized to a 2.0-kb mRNA in both the BALB/c *nu/nu* line and the G8 clone (Fig. 6) and to the J δ 1-hybridizing 4.8-kb rearranged band (Fig. 7).

Generation of a Distinct MHC-specific TCR- γ/δ -expressing CTL Line. The apparent TL-linked specificity and high degree of cross-reactivity of the BALB/c *nu/nu* anti-B10.BR TCR- γ/δ cell line appeared to contrast with the majority of TCR- α/β -expressing CTL previously described (36). In order to further explore the repertoire of TCR- γ/δ -bearing T cells, studies were undertaken to generate additional MHC-specific TCR- γ/δ cells. Lymph node cells from C57BL/10 (B10) *nu/nu* mice, primed in vivo with allogeneic B10.BR spleen cells, were restimulated in vitro as previously described (21). After 7 wk of culture the resulting cell line was analyzed for cell surface phenotype, antigen specificity and TCR expression. First, the B10 *nu/nu* anti-B10.BR line was shown to be CD3⁺, CD4⁻, CD8⁻ (data not shown) and cytolytic. The antigen specificity was therefore examined in cytolytic assays. As was previously observed for the BALB/c TCR- γ/δ CTL line, the B10 *nu/nu* CTL line was specific for an antigen localized to the *D*, *L*, *Qa*, or *TLa* regions of the MHC (Fig. 9). For example, the H-2^k-specific CTL lysed B10.BR (H-2K^k, D^k) and C3H.KBR (H-2K^b, D^k), but not B10 (H-2K^b, D^b) or B10.A(4R) (H-2K^k, D^b) target cells. However, in contrast to the BALB/c *nu/nu* TCR- γ/δ CTL line, the antigenic determinant recognized by the B10 *nu/nu* TCR- γ/δ CTL was not expressed on allogeneic target cells and did not map to the H-2TL gene loci. The CTL lysed C3H.KBR (H-2K^b, D^k, Q^k, TL^k) but not B6.K1 (H-2K^b, D^b, Q^k, TL^k). Therefore, the lytic activity of this CTL line maps to the *H-2D^k* locus, a specificity classically observed for TCR- α/β CTL.

The TCR complex expressed on this line was examined by two-dimensional non-reduced/reduced SDS-PAGE of radiolabeled immunoprecipitates (Fig. 10). Anti-CD3 mAb (*top panel*) immunoprecipitated a disulfide-linked TCR dimer (nonreduced mol wt 90×10^3 , reduced mol wt $45\text{--}47 \times 10^3$) distinct from the TCR- γ/δ proteins expressed by the BALB/c TCR- γ/δ CTL line and distinct from TCR- α/β proteins expressed by B10 spleen cells (data not shown). We have recently identified T cells

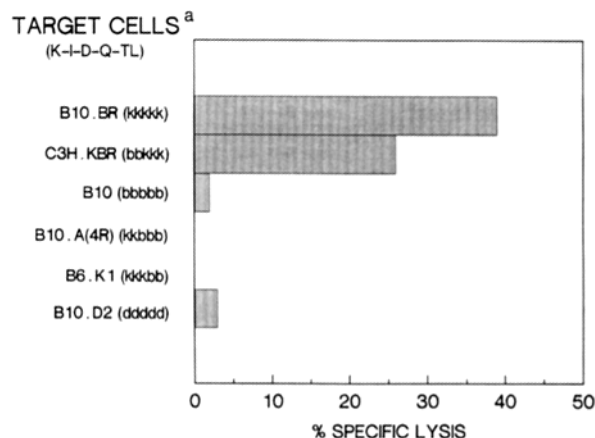


FIGURE 9. Lysis of target blast cells from MHC congenic mice by the C57BL/10 *nu/nu* anti-B10.BR CTL. (a) The MHC haplotypes (K-I-D-Q-TL) are: B10.BR (kkkkk), C3H.KBR (bbkkk), B10 (bbbbbb), B10.A(4R) (kkbb), B6.K1 (bbkkk), and B10.D2 (dddd).

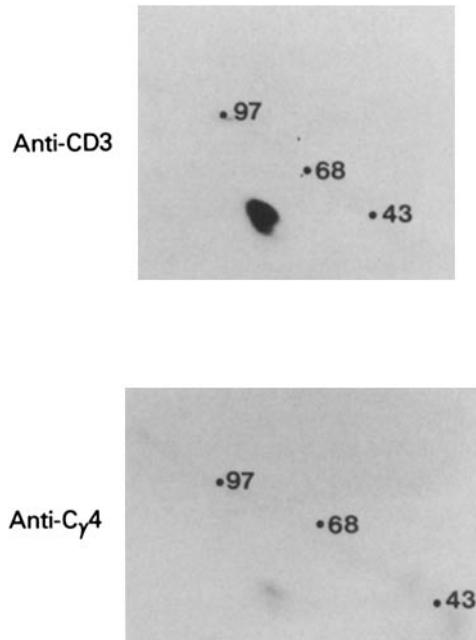


FIGURE 10. Detailed characterization of the TCR- γ/δ protein expressed on the B10 *nu/nu* anti-B10.BR CTL. Anti-CD3 mAb (*top*) and anti-TCR-C γ 4 antiserum (*bottom*) was used to immunoprecipitate radiolabeled CTL. The immunoprecipitates were analyzed by two-dimensional (nonreducing conditions along the horizontal dimension and reducing conditions along the vertical dimension) off-diagonal PAGE gels. Molecular weights are noted as dots on the figure.

in the periphery of normal B10 mice that express a TCR complex consisting of a TCR- δ chain disulfide-linked to the product of the *TCR-C γ 4* gene (5). Our studies using an antiserum developed against a peptide corresponding to the COOH-terminal region of the predicted C γ 4 gene product (37) precipitated at least two heterodimeric structures, one of which migrated at a molecular weight similar to the B10 anti-B10.BR line. Therefore, lysate of radiolabeled B10 anti-B10.BR CTL was immunoprecipitated with the anti-C γ 4 antiserum. As seen in the lower panel (Fig. 10), the antiserum immunoprecipitated the same TCR proteins as the anti-CD3 antibody (*upper panel*), consistent with the conclusion that this cell line expresses a TCR- γ/δ heterodimer and that the TCR- γ chain is encoded by a rearranged *V γ /C γ 4* gene.

Discussion

The identification of T cells that express TCR- γ/δ proteins has led to an active interest in determining the specificity, repertoire, and physiologic function of this novel T cell subset. Initial studies demonstrated that TCR- γ/δ T cells, isolated from peripheral T cell populations and activated with an array of antigen-independent stimuli such as mitogens or mAbs, develop non-MHC-restricted cytolytic activity (38, 39). For instance, CD4⁻CD8⁻ human T cell clones activated with Con A, IL-2, and allogeneic spleen cells lyse a variety of tumor targets including NK-sensitive targets (38). Thus, it has been suggested that the lymphokine-activated killer activity reported in activated peripheral lymphocytes may, in part, be mediated by T cells expressing the TCR- γ/δ receptor (40). It has also been suggested that TCR- γ/δ T cells recognize tumor antigens and function *in vivo* in tumor surveillance (39)

by recognizing non-MHC-restricted tumor target antigens. In fact, in some instances, the non-MHC-restricted lytic activity was inhibited by anti-CD3 or anti-TCR antibodies (38, 40). However, recent studies in our laboratory and others (41) have raised questions as to the role of the TCR- γ/δ receptor in mediating this activity. Therefore, our efforts have focused on generating antigen-specific TCR- γ/δ cells.

As an approach to identifying the ligand(s) and exploring the repertoire for T cells expressing TCR- γ/δ , we have established alloreactive T cell lines and clones from the peripheral lymph nodes of athymic *nu/nu* mice stimulated with allogeneic spleen cells. Two independent T cell lines have been described in this report. Both CTL lines have the CD3⁺,CD4⁻,CD8⁻ phenotype, express a TCR- γ/δ receptor, and recognize an MHC-linked class I antigen. However, the specificity and the receptor usage of the two CTL populations are distinct. One CTL line, and the clones derived from that line, expressed a TCR complex composed of the product of a rearranged *V γ 2/C γ 1* gene associated with a TCR- δ protein encoded by a well-characterized *V α* gene segment, *V α 11*, rearranged to both the *D δ 1* and the *J δ 1* variable gene elements. Functional analysis of the BALB/c *nu/nu* T cell line showed that it recognized a relatively nonpolymorphic MHC-linked class I molecule mapping within or to the right of the TL region of the MHC complex. Based on these initial results, it is interesting to speculate that the TCR- γ/δ cells have evolved to recognize a distinct subset of MHC antigens encoded by the *H-2Q* and *H-2TL* regions. In fact, the abundance of TCR- γ/δ cells in the epithelia of the skin may provide a unique role for these cells in the recognition of certain infectious agents presented in the context of non-MHC K, D, or L gene products or modified class I antigens. The TL or Q region-encoded class I antigens may be preferentially expressed on peripheral APC located in epithelia enriched for TCR- γ/δ cells. Alternatively, the presence of both TL antigens and TCR- γ/δ -expressing T cells early in thymus ontogeny may suggest a role for these antigens in thymic education of TCR- γ/δ or TCR- α/β cells. The fact that the MHC antigens that may be recognized (TL α or Q α) are less polymorphic may also explain the apparently limited *V γ* and *V δ* gene usage observed in the whole TCR- γ/δ population (10; Fig. 6). However, the demonstration of classical H-2D^k-specific class I MHC alloreactivity in the second γ/δ receptor expressing T cell line we have described suggests a potentially more diverse repertoire.

Sequence data available from the limited number of TCR- δ genes studied to date has revealed several that use known *V α* segments rearranged to *D δ* and *J δ* elements, although the majority appear to express a unique subset of *V δ* genes (19, 20). The expression of a *V α* gene segment by our MHC-specific γ/δ receptor-bearing T cells may have implications for the repertoire of these cells. Although the TCR- γ locus has limited germline diversity, the potential for extensive diversity of TCR- δ has already been suggested by the observation that the majority of full-length δ cDNAs derived from an adult thymocyte cDNA library encode sequences from both *D δ 1* and *D δ 2* gene segments, allowing for the addition of N region nucleotides at the three distinct junctions (V-D δ 1, D δ 1-D δ 2, and D δ 2-J δ). The expression of *V α* genes in γ/δ receptors could further amplify their potential diversity. In fact, if additional analysis indicates that the entire germline *V α* repertoire can participate in the formulation of functional TCR- δ genes, then the potential diversity of the TCR- γ/δ may be much greater than originally proposed based upon the limited TCR- γ germline repertoire and the apparent limited diversity of the expressed γ/δ receptors observed in unselected thymocyte and peripheral CD3⁺,CD4⁻,CD8⁻ T cell popula-

tions (10). These observations may be particularly pertinent in light of recent studies with TCR- β transgenic mice (42). These mice express only the TCR- β protein encoded by the transgene (endogenous TCR- β genes are incompletely rearranged) in association with a heterogeneous host-encoded TCR- α repertoire, and are nevertheless reported to manifest normal allogeneic responses. The TCR- γ/δ repertoire has the potential to be at least as diverse as the TCR- α/β repertoire of such transgenic animals. Moreover, the expression of a V_α gene in the first MHC antigen-specific TCR- γ/δ -bearing T cell invites speculation that this association may not be fortuitous. One striking difference between most V_α genes and the unique V_δ gene segments thus far isolated appears to be that unlike most V_α genes, V_δ genes represent single copy families (20). Perhaps TCR- γ/δ that express the unique V_δ segments recognize a distinct set of antigens and those TCR expressing V_α gene elements will comprise the majority of TCR- γ/δ cells with MHC-linked specificity.

Interestingly, the $V_{\alpha 11}$ gene segment of $\delta G8$ has been previously shown to be the predominant TCR- α gene expressed in a class II MHC-restricted antigen response (28, 35, 43). Thus, it is possible that TCR- γ/δ receptors have the potential to recognize a broad range of MHC-encoded ligands. We have shown here that whereas the BALB/c anti-B10.BR T cell clones appear to recognize a nonconventional class I MHC antigen encoded within the TL region, the B10 *nu/nu* anti-B10.BR γ/δ T cell line recognizes a conventional class I MHC molecule encoded within the H-2D^k subregion. It may be that additional TCR- γ/δ T cells will recognize class II MHC molecules as well as a variety of non-MHC peptides in an MHC-restricted fashion, analogous to TCR- α/β . Analysis of additional MHC-specific TCR- γ/δ -expressing T cell lines and clones is in progress to address these questions.

Summary

Analyses of TCR-bearing murine and human T cells have defined a unique subpopulation of T cells that express the TCR- γ/δ proteins. The specificity of TCR- γ/δ T cells and their role in the immune response have not yet been elucidated. Here we examine alloreactive TCR- γ/δ T cell lines and clones that recognize MHC-encoded antigens. A BALB/c *nu/nu* (H-2^d)-derived H-2^k specific T cell line and derived clones were both cytolytic and released lymphokines after recognition of a non-classical H-2 antigen encoded in the TL region of the MHC. These cells expressed the $V_\gamma 2/C_\gamma 1$ protein in association with a TCR- δ gene product encoded by a V_α gene segment rearranged to two D_δ and one J_δ variable elements. A second MHC-specific B10 *nu/nu* (H-2^b) TCR- γ/δ T cell line appeared to recognize a classical H-2D-encoded MHC molecule and expressed a distinct $V_\gamma/C_\gamma 4$ -encoded protein. These data suggest that many TCR- γ/δ -expressing T cells may recognize MHC-linked antigens encoded within distinct subregions of the MHC. The role of MHC-specific TCR- γ/δ cells in immune responses and their immunological significance are discussed.

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